

UNIVERSITY OF THESSALY

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**M.SC. "RESEARCH METHODOLOGY IN
BIOMEDICINE, BIOSTATISTICS AND CLINICAL
BIOINFORMATICS**

MASTER THESIS

**AN OBSERVATIONAL, PROSPECTIVE STUDY
FOR ASSESSING THE IOP-LOWERING
EFFICACY AND OCULAR SURFACE
TOLERABILITY OF TRAVOPROST WITH sofZia
IN PATIENTS WITH OPEN-ANGLE GLAUCOMA
OR OCULAR HYPERTENSION**

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1. INTRODUCTION

1.1. Background

Intraocular pressure (IOP) is an important risk factor for the development and progression of glaucoma. In recent years, The Ocular Hypertension Treatment Trial has demonstrated that IOP reduction can prevent the development of glaucoma among individuals with ocular hypertension and can reduce the risk of glaucoma progression among subjects with both normal and elevated IOP. The impact of both short-term and long-term IOP variability on progression risk has also been explored, with many (but not all) studies finding a positive relationship between greater IOP variability and higher rates of glaucomatous progression or development.

Treatment of glaucoma aims on the reduction of intraocular pressure (IOP). While several laser and surgical therapies are available, topical medication continues to be a commonly utilized initial treatment option. Due to their once daily dosing, excellent efficacy, and favourable side effect profile, the prostaglandin analogues are frequently chosen as the first line medication for the reduction of IOP in most forms of glaucoma and ocular hypertension. It is believed that prostaglandin analogues lower IOP primarily by increasing aqueous outflow through the uveoscleral pathway. Based on more recent evidence, these medications may also augment the traditional outflow pathway through the trabecular meshwork and Schlemm's canal. There are currently several molecules within the prostaglandin analogue class that are commercially available with each having a distinct profile for pressure lowering and tolerability.

1.2. Travoprost

Travoprost ophthalmic solution 0.004% is a prostaglandin analog available throughout the global marketplace and indicated for IOP reduction in subjects with open-angle glaucoma or ocular hypertension. Travoprost ophthalmic solution has been formulated with a variety of preservatives.

Mechanism of action

Travoprost, like the other prostaglandin analogues latanoprost and bimatoprost, is a synthetic analogue of prostaglandin F_{2α}. Prostaglandins are a family of molecules found ubiquitously throughout most tissues and organs. They are synthesized enzymatically from fatty acids, and all contain 20 carbon atoms, including 5 in a ring formation. Their functions are diverse, and include roles in muscle constriction, inflammation, and platelet aggregation. These various functions are mediated by binding of specific prostaglandins to one or more of numerous prostaglandin receptors. The prostaglandin receptors are transmembrane, G-protein-coupled receptors.

The travoprost molecule is an ester pro-drug that is hydrolyzed by corneal esterases into its active free-acid form. The IOP-lowering efficacy of all three prostaglandin

analogues is dependent upon interaction with the prostaglandin FP receptor, as evidenced by the lack of IOP reduction seen with these drugs in eyes of FP receptor-deficient mice. Once hydrolyzed in the eye, travoprost acid then binds to prostaglandin FP receptors in both the ciliary muscle and the trabecular meshwork. In cultured cells from both ciliary muscle and trabecular meshwork in human, rat, and mouse models, travoprost acid exhibits higher binding affinity and higher potency at the FP receptor, and also demonstrates higher selectivity for the FP receptor than for other prostaglandin receptors, than either latanoprost or bimatoprost FP-receptor binding by prostaglandin $F_{2\alpha}$ and its analogues results in numerous physiologic responses within ciliary muscle cells. These include phosphoinositide turnover, intracellular Ca^{2+} mobilization, and mitogen-activated protein (MAP) kinase activation. In addition, FP receptor activation indirectly stimulates formation of cAMP via activation of the coupled G-protein by stimulating the synthesis of PGE_2 . This in turn leads to increased cellular levels of c-Fos and c-Jun within the nuclei of ciliary smooth muscle cells. These two proteins can heterodimerize, forming a complex that binds to the promoter regions of some genes, thus promoting their transcription. The results of these FP receptor-mediated intracellular signals include increased production of several matrix metalloproteinases (MMPs), specifically MMP-1, -2, -3 and -9, in cultured human ciliary smooth muscle cells. MMPs are a family of enzymes that are capable of degrading all extracellular matrix components, including collagen. In monkey eyes, topical exposure to prostaglandin $F_{2\alpha}$ reduces collagen types I, II, and IV within the ciliary muscle. Remodeling of the extracellular matrix of the ciliary body is hypothesized to lower IOP by creating or increasing spaces between the ciliary muscle fiber bundles, thus increasing outflow through the uveoscleral pathway.

[Travoprost and preservatives](#)

Travoprost (Travatan, Alcon, Fort Worth, TX) was first approved by the Food and Drug Administration (FDA) in 2001. The multi-dose bottle for travoprost available in the United States was originally preserved with the detergent preservative benzalkonium chloride (BAK). This formulation has been previously shown to significantly lower IOP during both the diurnal and nocturnal periods in patients with open angle glaucoma and ocular hypertension. A report by Sit et al has demonstrated a durable IOP lowering response of travoprost with BAK for 41 to 63 hours after last dose. Despite its efficacy and widespread use in ophthalmic medications, chronic use of BAK can have several negative effects on ocular tissues in specific patient populations. Prolonged BAK exposure in cell culture models results in arrest of cell growth, apoptosis, and even necrosis at very high doses. These detrimental effects are implicated in ocular surface disease that is frequently present in patients taking multiple BAK-preserved medications. In 2006, BAK was removed from travoprost and replaced with a novel ionic-buffered preservative system called sofZia (Travatan Z, Alcon). After application on the ocular surface, sofZia components break up into innate ingredients with the theoretical benefit of decreased hyperemia and improved tolerability, although results from published studies are conflicting.

1.3. Rationale for the study

The decision to select medical therapy options relies primarily on the efficacy of the available medications in various therapeutic trials. Sadly, most published studies only evaluate the short-term, daytime efficacy of medical therapy. Travoprost is increasingly becoming a popular first-line monotherapy option for POAG patients as there is cumulative evidence suggesting good quality of short term 24-hour IOP control with low fluctuation, especially with evening administration. As POAG is a 24-hour, lifelong disease, an ideal antiglaucoma medication should be successful in reducing IOP during day and night. Importantly, 24-hour studies have confirmed that 24-hour IOP monitoring can significantly modify individualized glaucoma care in many glaucoma patients. This study will examine whether the IOP-reduction of travoprost is achieved at the 1600-hour time point which approaches the end of the dosing interval.

2. TRIAL OBJECTIVES

The primary objective is to evaluate the IOP-lowering efficacy of travoprost with sofZia used as a first-line therapy during a 12-weeks follow-up period

Secondary objectives:

- evaluation of subjective symptoms (ie, burning, foreign-body sensation, itching, and stinging)
- evaluation of objective clinical signs such as conjunctival hyperemia, iris color darkening and eyelash darkening, thickening and lengthening, localized pigmentation of conjunctiva, iris and choroid

3. STUDY DESIGN

This is an observational, prospective study for Travoprost with sofZia in patients with open-angle glaucoma or ocular hypertension. A total of 165 newly diagnosed patients with open-angle glaucoma or elevated intraocular pressure will be enrolled and will be followed up for 12 weeks after starting them on travoprost 0.004% BAK-free once daily. The initiation of this treatment modality will be based on the decision of the physician only according to the clinical practice.

4. SUBJECT SELECTION

4.1. Inclusion criteria

This study will enrol newly diagnosed, previously untreated POAG patients who exhibit characteristic glaucomatous disc damage and visual field loss. Subjects must meet all of the following inclusion criteria to be eligible for enrolment into the study:

- age above 45 years
- no previous ocular surgery other than uncomplicated phacoemulsification cataract surgery performed at least 6 months before entering the study
- open-angle by gonioscopy (grades III and IV according to the Shaffer classification)
- untreated IOP between 21 and 35 mm Hg at 10:00 AM \pm 1 hour verified by 2 separate measurements (enrollment IOP)
- signed informed consent

4.2. Exclusion criteria

Subjects presenting with any of the following exclusion criteria will not be included in the study:

- any type of glaucoma other than POAG
- corneal or other anatomic conditions making applanation tonometry unreliable
- central corneal thickness ≤ 500 μm or ≥ 600 μm
- signs or history of macular edema, herpetic, or other ocular inflammatory disease
- history of allergy or adverse event related to the study medication
- history or suspicion of poor adherence to medical therapy
- best-corrected visual acuity < 0.4 (Snellen)
- cup-to-disc ratio > 0.7
- mean deviation (MD) worse than -15 dB in Humphrey 24-2 SITA standard perimetry (Humphrey Visual Field Analyzer; Carl Zeiss Meditec Inc., Dublin, CA)
- the possibility of significant visual deterioration and optic nerve damage due to participation to the study according to the principal investigator's judgment.

- patients who are pregnant or who can become pregnant during the study
- patients with IOP higher than 30 mmHg at baseline visit or those who had undergone glaucoma filtering surgeries

Patients will be also excluded if they are unable to understand the study procedures, give informed consent, or are using systemic corticosteroids.

5. STUDY PROCEDURES

During an initial eligibility visit, all qualified patients will undergo a detailed ophthalmic examination and their ocular and systemic clinical data will be recorded. Study participants will be admitted at the Ophthalmologic Department of the University General Hospital of Thessaloniki, AHEPA for an untreated 24-hour IOP evaluation (baseline visit) with Goldmann tonometry. The IOP will be recorded at 10:00 AM, 2:00 PM, 6:00 PM, 10:00 PM, 2:00 AM, 6:00 AM, and 10:00 AM (± 1 h). The day before the 24-hour IOP assessment, a reliable 24-2 SITA standard visual field test will be performed (Humphrey Visual Field Analyzer; Carl Zeiss Meditec Inc.).

Subsequently all study patients will be assigned to topical therapy with travoprost 0.004% eye-drops with sofZia (Travatan Z, Alcon Laboratories Inc.) administered once in the evening (8:00 PM) in both eyes in accordance with current clinical practice. To ensure that the target IOP will be reached, according to the principal investigator's judgment, a safety visit will be performed approximately 4 weeks after treatment initiation. The predetermined range of target IOP reduction selected in this study with travoprost monotherapy will range between 20% and 30%. After this visit, all patients will be scheduled for a visit at 12 weeks after the baseline visit at 16:00 AM ± 1 hour. This schedule of follow-up will be maintained for the whole duration of the trial. A comprehensive clinical examination will be performed at each visit recording the possible subjective symptoms and objective clinical signs. Enrolled patients will continue on travoprost monotherapy as long as their target IOP will be successfully maintained. Calibrated Goldmann tonometers will be used throughout the duration of the trial by a clinician masked to the previous baseline or treated IOP data.

5.1. Assessment of study endpoints

Intraocular pressure reduction

Intraocular pressure will be measured and recorded at each visit using Goldmann applanation tonometry.

Subjective symptoms

Subjective symptoms will be evaluated using a four-point scale ranging from "no symptoms," "mild symptoms," "moderate symptoms" to "severe symptoms."

Iris color darkening and eyelash darkening, thickening and lengthening

Investigators will take photographs of the anterior surface of the eyes (including iris, conjunctiva and eyelashes) at baseline and follow-up visits, allowing objective assessments of the recorded changes of iris colour darkening, eyelash darkening, thickening and lengthening since baseline.

The baseline colour of the iris will be assessed by an ophthalmologist and is classified into one of the five groups: homogeneously brown, blue brown, gray brown, green brown and yellow brown.

Possible degrees of iris colour darkening at follow-up visits will be assessed by the ophthalmologist. The change will be classified into one of six categories:

- Decrease- a reduction or lightening of pigmentation
- No change- no visible change in pigmentation recorded
- Slight- a slight but clearly visible increase in pigmentation
- Moderate- the increase in pigmentation is more than slight but not striking
- Marked- a strong increase in pigmentation
- Not evaluable

Possible degrees of eyelash darkening/thickening at follow-up visits will be classified by the ophthalmologist into one of six categories as described above (i.e. decrease, no change, slight increase, moderate increase, marked increase and not evaluable)

The longest eyelash (mm) measured by calliper or ruler will be recorded at baseline and each follow-up visit.

Localized pigmentation of conjunctiva, iris and choroid

The anterior surface photographs can be used to assess pigmentation of conjunctiva and iris. Investigators will also take retinal fundus photographs at baseline and follow-up visits, allowing objective assessment of the recorded changes in choroid pigmentation. The previous existence of nevi or freckles of conjunctiva, iris and choroid will be recorded at baseline (yes/no). Possible degrees of change at follow-up visits will be recorded by the ophthalmologist and will be classified into one of six categories:

- Increased number
- Increased size (height, length and width)
- Changing colour (darkening or lightening)
- Changing uniformity of the colour (increase or decrease)
- Increased irregularity of the border/decreased sharpness of the border
- No change

Conjunctival hyperemia

Conjunctival hyperemia will be assessed and graded by ophthalmologist at baseline and follow-up visits as follows:

- | | |
|--------------|---|
| 0 = None | Normal: few vessels of palpebral or bulbar conjunctiva easily observed |
| 1 = Mild | Reddening of the palpebral or bulbar conjunctiva |
| 2 = Moderate | Bright reddening of the palpebral or bulbar conjunctiva |
| 3 = Severe | Deep, bright and diffuse reddening of the palpebral or bulbar conjunctiva |

5.2. Subject withdrawal

Subjects or legally accepted representatives may withdraw from the study at any time at their own request, or they may be withdrawn at any time at the discretion of the ophthalmologist or sponsor for safety, behavioral, or administrative reasons. In any circumstance, every effort should be made to document subject outcome, if possible. The ophthalmologist should inquire about the reason for withdrawal and follow-up with the subject regarding any unresolved adverse events.

If the subject withdraws from the study, and also withdraws consent for disclosure of future information, no further evaluations should be performed, and no additional data should be collected. The sponsor may retain and continue to use any data collected before such withdrawal of consent.

6. STATISTICAL CONSIDERATIONS

The primary endpoint of the study is the mean IOP reduction achieved after 12 weeks of treatment with Travoprost with sofZia monotherapy. For this reason mean IOP at baseline will be estimated as well as mean IOP at endpoint i.e. 12 weeks. Both eyes per patient are included in the analysis.

6.1. Sample size determination

In order to estimate the appropriate sample size needed, t-test dependent sample will be used. As proposed by Harvey B Dubiner and Robert Noecker in Clin.Ophthalmol. 2012; 6: 525–531 mean IOP reduction by 12th week was estimated to be -7.6 mmHg (-7.6 +/- 3.3)

Using G Power 3.0.10. and given that $\alpha=0.05$ and power $(1-\beta)=0.8$ we have the following output parameters:

Noncentrality parameter $\delta=-2.8$

Critical t= -1.976

Df= 149

Total sample size= 150

Taking into consideration that an expected dropout we have to increase this number by 10%. Finally, the sample size needed is a number of 165 patients.

6.2. Statistical Analysis

The target of the present study will be to estimate the mean IOP reduction achieved by the 12th week in patients treated with travoprost monotherapy. For this reason, mean IOP at baseline and mean IOP at endpoint (12th week- primary outcome) at 16:00 AM will be calculated and then compared with each other using t-test dependent samples and 95% Confidence Interval.

In addition demographic characteristics will be described as age, sex, ethnicity, co-existent diseases (hypertension, diabetes mellitus).

As for the secondary outcomes, mean IOP at 4-week visit will be estimated and compared to mean IOP at baseline using t-test dependent samples. Furthermore, statistics will present the occurrence of subjective symptoms and objective clinical signs, as ocular hyperemia, iris colour darkening and eyelash darkening, thickening and lengthening, and localized pigmentation of conjunctiva iris and choroid.

7. ADVERSE EVENT REPORTING

7.1. Adverse events

All observed or volunteered adverse events regardless of suspected causal relationship to travoprost will be recorded.

For all adverse events, the ophthalmologist must pursue and obtain information adequate both to determine the outcome of the adverse event and to assess whether it meets the criteria for classification as a serious adverse event. For all the adverse events sufficient information should be obtained by the ophthalmologist to determine the causality of the adverse event. The ophthalmologist is required to assess causality. For adverse events with a causal relationship to travoprost, follow-up by the ophthalmologist is required until the event or its sequelae resolve or stabilize at a level acceptable to the ophthalmologist.

7.2. Reporting period

For serious adverse events, the reporting period begins from the time that the subject provides informed consent, which is obtained prior to the subject's participation in the study, through the entire study period or 28 calendar days after the last administration of travoprost within the observational period, whichever is latest.

7.3. Definition of an Adverse Event

An adverse event is any untoward medical occurrence in a clinical investigation subject administered a product or medical device: the event needs not necessarily have a causal relationship with the treatment or usage. Adverse events may include:

- Abnormal test findings
- Clinically significant symptoms and signs
- Changes in physical examination findings
- Hypersensitivity
- Progression/worsening of underlying disease

Additionally, they may include signs or symptoms resulting from:

- Drug overdose
- Drug abuse
- Drug misuse
- Drug interactions
- Drug dependency
- Exposure during pregnancy

8. COMMITTEES

8.1. Executive Committee

The EC consists of members of the academic leadership of the study and 1 member from the sponsoring company. The EC will ultimately be responsible for the conduct of the study including addressing any Data Monitoring Committee recommendations and overseeing publication of the results.

8.2. Steering Committee

A Steering Committee will be formed consisting of members who are lead investigators from each country/region. The Steering Committee will advise and assist the EC with regard to the scientific and operational aspects of the study.

8.3. Independent Data Monitoring Committee

This study will be conducted under the auspices of an independent Data Monitoring Committee (DMC), which will monitor the progress of the study and ensure that the safety of subjects enrolled in the study is not compromised. The DMC will have a chairperson and include at least 2 cardiologists, a neurologist, as well as a statistician. This committee will review accumulating data on a regular basis, and may request to review partially unblinded or unblinded accumulating data. The DMC will make recommendations to the Executive Committee and Sponsor regarding the continuing safety of subjects currently enrolled and yet to be enrolled in the trial. At all times during the course of the study, the DMC may request access to unblinded data if needed.

8.4 Clinical Endpoint Committee

The Clinical Endpoint Committee (CEC), composed of experts in the relevant fields, will review, in a blinded manner, all reported study outcomes to provide consistency and validity in the assessment of outcomes. Their decisions will be based on blind clinical data. Their decisions will be used for the final statistical analyses

9. ETHICAL CONSIDERATIONS

This study will be conducted in compliance with the protocol, the ethical principles set forth in the Declaration of Helsinki, the ICH Guideline E6 for GCP and applicable regulatory requirement(s). Good clinical practice (GCP) is an international ethical and scientific quality standard for designing, conducting, recording, and reporting research studies that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety, and well-being of study

subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki, and that the clinical study data are credible.

9.1. Institutional Review Board/Independent Ethics Committee

The protocol and any amendments, the Investigator's Brochure, the subject informed consent and any information on compensation for study-related injuries or payment to subjects, will receive IRB/IEC approval prior to initiation of the study. During the study the investigator will send to the IRB any reports of adverse events that are serious, unlisted, and associated with the investigational drug and any new information that may adversely affect the safety of the subjects or the conduct of the study.

Study personnel involved in conducting this study will be qualified by education, training, and experience to perform their respective task.

9.2. Informed Consent

Before a subject's participation in the study, it is the Investigator's responsibility to obtain freely given consent, in writing, from the subject or a legally acceptable representative after adequate explanation of the aims, methods, anticipated benefits, and potential hazards of the study and before any protocol-specific procedures or any study drugs are administered.

The written ICF should be prepared in the local language(s) of the potential subject population. The informed consent should be approved by the IRB prior to being provided to potential subjects.

The written informed consent form and any other written information to be provided to subjects should be revised whenever new information becomes available that may be relevant to the subject's consent. Any revisions to the written informed consent form and/or to other written information provided to the subject should be approved by the responsible IRB in advance of use.

Subjects unable to give their written consent (e.g., stroke subjects, or subjects with severe dementia) may only be enrolled in the study with the consent of a legally acceptable representative. The subject must also be informed about the nature of the study to the extent compatible with the subject's understanding.

If a subject or a subject's legally acceptable representative is unable to read, an impartial witness should be present during the entire informed consent discussion.

Subjects may withdraw consent from participation in the study at any time. In the event a subject withdraws consent to receive study drug, the site may (with the subject's agreement) continue to contact the subject, general practitioner, and any other physician or medical care provider for the collection of outcome and survival follow-up data.

9.3. Subject Confidentiality

The Investigators and the Sponsor will preserve the confidentiality of all subjects taking part in the study, in accordance with GCP and local regulations.

The Investigator must ensure that the subject's anonymity is maintained. On the eCRFs or other documents submitted to the Sponsor or the CRO, subjects should be identified by a unique subject identifier as designated by the Sponsor. Sponsor personnel whose responsibilities require access to personal data should agree to keep the identity of study subjects confidential.

10. REFERENCES

Harvey B Dubiner, Robert Noecker Sustained intraocular pressure reduction throughout the day with travoprost ophthalmic solution 0.004% Clin. Ophthalmol. 2012; 6: 525–531.

Konstas AG, Mikropoulos D, Haidich AB, et al..Twenty-four-hour intraocular pressure control with the travoprost/timolol maleate fixed combination compared with travoprost when both are dosed in the evening in primary open-angle glaucoma. Br J Ophthalmol. 2009;93:481–485

Konstas AG, Mikropoulos D, Kaltsos K, et al..24-hour intraocular pressure control obtained with evening- versus morning-dosed travoprost in primary open-angle glaucoma. Ophthalmology. 2006;113:446–450

Przydryga JT, Egloff C.Intraocular pressure lowering efficacy of travoprost. Eur J Ophthalmol. 2004;14:416–422

Fechtner RD, Godfrey DG, Budenz D, et al. Prevalence of ocular surface complaints in patients with glaucoma using topical intraocular pressure-lowering medications. Cornea. 2010;29(6):618–621

Whitson JT, Trattler WB, Matossian C, Williams J, Hollander DA. Ocular surface tolerability of prostaglandin analogs in patients with glaucoma or ocular hypertension. J Ocul Pharmacol Ther. 2010;26(3):287–292.

Gandolfi S, Paredes T, Goldberg I, et al. Comparison of a travoprost BAK-free formulation preserved with polyquaternium-1 with BAK-preserved travoprost in ocular hypertension or open-angle glaucoma. Eur J Ophthalmol. 2012;22(1):34–44.

Gross RL, Peace JH, Smith SE, et al. Duration of IOP reduction with travoprost BAK-free solution. J Glaucoma. 2008;17(3):217–222

Dubiner HB, Noecker R. Sustained intraocular pressure reduction throughout the day with travoprost ophthalmic solution 0.004%. Clin Ophthalmol. 2012;6:525–531.

Stewart WC, Konstas AG, Nelson LA, Kruff B. Meta-analysis of 24-hour intraocular pressure studies evaluating the efficacy of glaucoma medicines. Ophthalmology. 2008;115(7):1117–1122

Horsley MB, Kahook MY. Effects of prostaglandin analog therapy on the ocular surface of glaucoma patients. Clin Ophthalmol. 2009;3:291–295.

Uusitalo H, Chen E, Pfeiffer N, et al. Switching from a preserved to a preservative-free prostaglandin preparation in topical glaucoma medication. *Acta Ophthalmol.* 2010;88:329–336.